



Effect of ethanol extract and alkaloidal fraction of *Ziziphus mauritiana* (Fam-Rhamnaceae) roots on fertility and sexual behavior of male wistar albino rats

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Abstract

Ziziphus mauritiana is known to cure sexual weakness, and general debility in traditional and folklore medicines. To evaluate the fertility effect of *Z. mauritiana* (ZM) root ethanol extract and alkaloidal fraction at the dose level of 50 mg/kg and 100mg/Kg of body weight administered orally in adult male rats, for 30 days, during this the rats were studied for sexual behavior and then they were euthanized and their serum level of testosterone and sperm count were measured. The treated animals score heavily over control group in all behavioral activities as attraction toward females, PEI (penile erection Index), Mount intromission while IL (Intromission latency) and PEL (Post ejaculation latency) were significantly ($p < 0.01$) reduced. A dose dependent increase in the serum levels of testosterone and sperm count was detected in treated rats which is more pronounced in alkaloidal fraction treated group (1.14 ± 0.17 ng/ml) as compared to control testosterone levels (0.93 ± 0.15 ng/ml) and epididymal sperm count of (11.55 ± 0.55 million/mL) as compared to control group (7.87 million/mL) ($p < 0.01$). Thus this study established that ethanolic extract and alkaloidal fraction of ZM roots has a positive impact on male reproductive functions or fertility but alkaloidal fraction has more pronounced effect. So **activity is due to presence of alkaloids**.

KEY WORDS: ZIZIPHUS MAURITIANA, ANDROGENIC, MALE RAT, TESTOSTERONE

Introduction

Z. mauritiana is well known plant in Ayurvedic literature. *Z. mauritiana* a tropical fruit tree species. It is known as chinee apple, chinese date, cottony jujube, Indian cherry, Indian jujube, Indian plum, jujube in English, jujubier, massonnier, prune Saint-Paul in French and baher, bahir in Hindi etc. It is a spiny, evergreen shrub or small tree up to 15 m high, with trunk 40 cm or more in diameter; spreading crown; stipular spines and many drooping branches¹.

Root extract of *Z. mauritiana* posses antidiarrhoeal activity in rodents in castor oil induced diarrhoea². Aqueous extract of *Z. mauritiana* leaf can be used for the prevention and treatment of fatty liver, atherosclerosis and other diseases associated with high levels of cholesterol and triglyceride and pretreatment was found to confer more protection than co-treatment, hence pretreatment should be preferred. ³ The methanolic extract of *Z. mauritiana* stem bark posses antiulcer activity. ⁴ Investigation of the MeOH extract revealed that isolated alkaloids exhibited potent antiplasmodial activity against the parasite *Plasmodium falciparum*. ⁵ *Ziziphus mauritiana* is used to cure sexual weakness and general debility in India since ancient time and also to worship Lord Shiva (lord of fertility) in the arrival of spring season or mating season. Thus here we aim to investigate the use of *Ziziphus mauritiana* roots in fertility and sexual behaviour of male wistar albino rats.

Methods

Plant material

Dried roots were collected from deer park, Moradabad in Feb 2010 and identified by Dr DV Amla, of National Botanical Research Institute (NBRI), Lucknow, Uttar Pradesh, India, and a voucher specimen no. (NBRI-SOP- 202) was preserved in NBRI for future references.

Chemicals

Ethanol was purchased from CDH, New Delhi,

Testosterone was marketed preparation Testoviron Depot (German Remedies).

Preparation of extract

Ethanollic extract

The dried roots were crushed to moderately coarse powder (60–80 mesh size) and macerated with 50% aqueous ethanol for seven days and then filtered and solvent was removed by vacuum under pressure the yield was 13% w/w.

Alkaloidal fraction

The powered plant material (10 g) was homogenize with MeOH-H₂O(4:1) for 5 min in 10 times volume of weight then it was filtered, evaporate to 1/10 of volume acidify with 2MH₂SO₄ then extract with three times volume of chloroform then aqueous acid layer was separated and basified to pH 10 with NH₄OH then extract with chloroform for getting alkaloidal⁶. And the extract was further dried by vaccum under pressure and the yield was 7% w/w.

Phytochemical Screening

For experiment, ethanolic root extract and alkaloidal fraction was characterized by performing various microchemical tests and TLC on precoated silica gel G plate (10×10) (E. Merk, Germany) developed in nButanol:acetic acid:water (5. 5:1. 5:3. 0 v/v) as mobile phase gave best resolution after derivatization with Dragendroffs reagent and [alkaloidal fraction of *Z. mauritiana*](#) was also characterized by HPTLC.

Procedure for HPTLC

Sample was centrifuged at 3000 rpm for 5 min. This solutions were used as test solution for analysis. Three microlitre of the above test solutions was loaded as 8 mm band length in the 5 x 10 Silica gel 60F254 TLC plate using Hamilton syringe and CAMAGLINOMAT5 instrument and then plate was developed in solvent system nButanol:acetic acid:water in ratio 5. 5:1. 5:3. 0 and then derived by Dragendroffs reagent and scanned at 254 nm.

Animal Stock

Male wistar albino rats and female wistar albino rats weighing 120–150 g were fed on standard diet and water *ad libitum*. The animals were housed at room temperature ($24 \pm 2^\circ\text{C}$) on a reversed day-night cycle (dark from 06:00 to 18:00). The animals were handled according to CPCSEA Guidelines of Good Laboratory Practice.⁷ The research protocol of the animal experimentation (Reg no. 837/ac/04/CPCSEA; Resolution no. 05/837ac/PH/10 of December 12, 2010) was approved by the 'Institutional Animal Ethical Committee' of College of Pharmacy, IFTM, Moradabad-244001, Uttar Pradesh, India.

Acute toxicity studies

Sterilized extract and alkaloidal fraction were administered intraperitoneally in water for injection to rats, and the dose that killed 50% of the animal population was estimated as the LD₅₀.⁹

The test substances was administered in a single dose. Animals were fasted 3 h prior to dosing water. And test substances were administered orally at a dose of 50, 100, 150, 250, 300, 350, 400, 450 and 500mg/kg. After the administration of test substance, food for the rats was withheld for 2 h.

Animals were observed individually after at least once during the first 30 min, periodically during the first 24 h, with special attention given during the first 4 h, and daily thereafter, for a total of 14 days. All the rats were observed at least twice daily with the purpose of recording any symptoms of ill-health or behavioral changes.

Signs recorded during acute toxicity studies

Direct observation parameters include tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma and death. The number of survivors was noted after 24 h and then these were maintained for a further 14 days with a daily observation.

Experimental design

Six adult males albino rats (wistar albino strain- 2. 5 months old) weighing between 120-150 g were kept in a cage for study for each groups. Six adult

female albino rats (wistar albino strain- 2. 0 months old) weighing between 120-150 g were kept at separate cages and allowed to enter in cages of male during sexual activity observation period. The extract was administered orally to the male rats with the help of micropipette. Extract and alkaloidal fraction was dissolved in sterile water at dose of 1mg/mL. The experiment was conducted during March 2011 for 30 days.

See Table 1.

Sexual behavior analysis

Male rat was placed in the observation glass chambers in order to acclimatize it with the cage environment. Sexually receptive female rat was then allowed to enter the test cage silently from a side door inside the cage. The behavioral observations were carried out taking into account the following parameters.^{10,11}

Mounting Behavior – It was determined and characterized by following parameters.

(A) Mount frequency – Average number of mount during 30 min observation.

(B) Mount latency- The leg time from the introduction of female in the cage to first mount.

Intromission Behavior – It was evaluated as follows.

(A) Intromission frequency – average number of Intromission during 30 min observation.

(B) Intromission latency- Intromission latency (IL) was considered as the time for first intromission after introduction of female in the cage.

(C) Post Ejaculation latency- After ejaculation time taken by rat for next intromission.

Penile Erection Index

Penile Erection (PEI) was determined when the rats bent down to lick their erect penis during the observation period. *Penile erection index (PEI)* was determined by multiplying the percentage of rats exhibiting at least one episode of penile erection

during 30-min observation period with the mean number of penile erections ^{10,11}

$$PI = \frac{\% \text{ of rats exhibiting penile erection} \times \text{Mean number of erections}}{\text{Mean number of erections}}$$

Tissue and blood sample collection

By the end of each treatment, all rats were exposed to mild ether anesthesia. Then blood (1.0 mL) was collected using a syringe and needle directly by cardiac puncture and allowed to clot in the 15 ml centrifuge tube. Reproductive organs, such as testes, epididymis, prostate, and seminal vesicle were isolated from the sacrificed rats and stored in deep freezer at -20°C.

Hormonal assays

Testosterone hormone concentration of male rats of each group was measured by quantitative analysis using HPLC with UV-Detector. ¹² An isocratic high-performance liquid chromatographic method for the determination of testosterone (T) (20 µL injection) and the optimum separation is achieved using a stationary phase of Octadecylsilyl silica gel column and mobile phase of water-Methanol (45:55, v/v) at flow rate of 1.0 mL/min and retention time of 18 minutes and UV-absorbance detection at 245 nm.

Sperm collection and analysis

For counting spermatozoa left and right epididymes of four rats of each group were homogenized and taken into 5 ml of 1% sodium citrate solution, squashed thoroughly with the help of needle and forceps until a milky suspension was obtained. The suspension was filtered through 80 µ mesh and the final volume made up to 10 ml. The made up volume was inclusive of washings of the filter. The suspension was thoroughly shaken and the spermatozoa were counted using a hemacytometer. The average numbers of sperms determined in every group are reported. ¹³

Results

Phytochemical Screening

Preliminary phytochemical screening for the presence of alkaloids, flavonoids, tannins, glycosides, resins, phenols, volatile oils and saponins were carried out using standard test procedures. ¹⁴

Ethanol extract shows presence of alkaloids, steroids, carbohydrates and phenolics. While alkaloidal fraction showed presence of alkaloids only. The chromatography of extract shows six separations in nButanol:Water:Acetic acid solvent system.

The chromatography of alkaloidal fraction showed four separations in nButanol: Water: Acetic acid in r=5.5:3.0:1.5 in UV light under 366nm wavelength. And its HPTLC profile shows presence of ten compounds.

See Figure 1.

The alkaloidal fraction shows presence of ten alkaloids in which the alkaloid with R_f value 0.33 is found in maximum concentration. Generally alkaloids are associated with fertility potentials. ¹³

Toxicity Studies

The LD₅₀ of the extract was estimated to be 450 mg/kg (ip) and for alkaloidal fraction it was 400mg/Kg(ip) of body weight in rats for study of 14 days.

See Table 1.

This study showed that there is significant enhancement in attraction toward females in male wistar albino rats as compared to control group animals and penile erection index and mount intromission also increase and post ejaculatory latency and intromission latency decreased that showed enhanced sexual per-

formance of extract and alkaloidal fraction treated animals.

See Table 2.

See Table 3.

Discussion

There was a significant increase ($p < 0.01$) in serum levels of testosterone of the male rats treated with extract as well as alkaloidal fraction of *Z. mauritiana* roots. When compared with the control group (Table 3). Elevation in serum testosterone level might be accounted for enhanced secretion of androgen resulted from adequate sperm production in both testicles and epididymis. It might be a probable cause for testicular and epididymal function as a result of androgen availability. Hence, sperm production in both organs were affected in treated male rats.

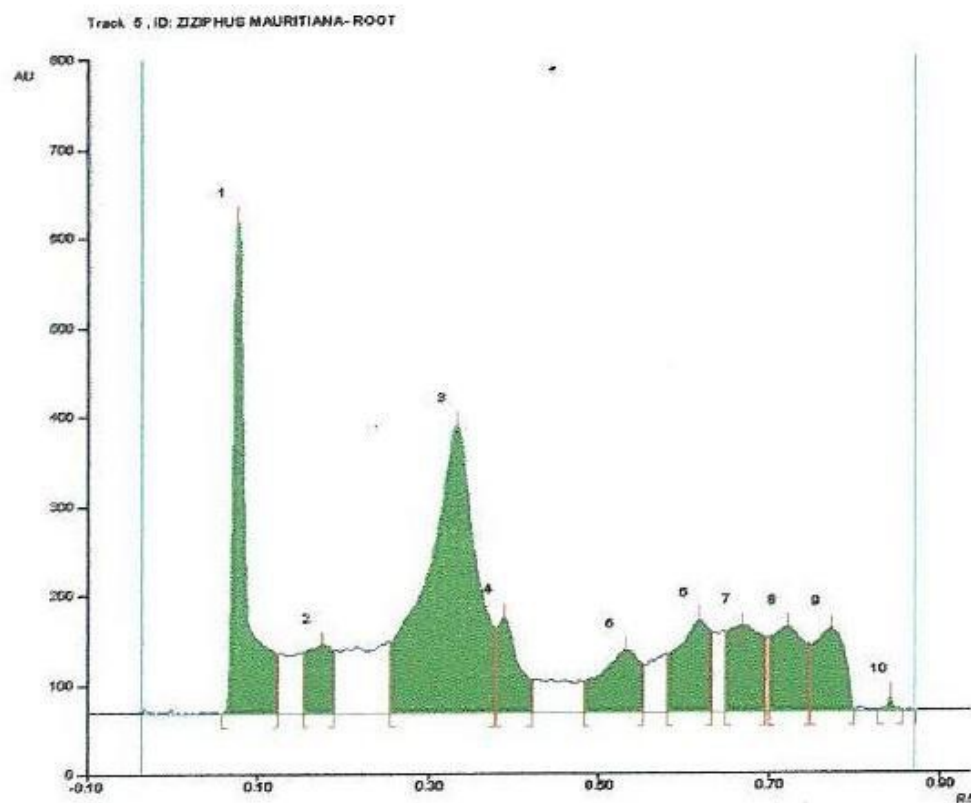
This study showed that both ethanol extract and alkaloidal fraction of *Z. mauritiana* root induced dose dependent increase in functional fertility and enhanced sexual activity in male wistar albino rats. The potential lies in alkaloidal extract and ten alkaloids are present in the fraction. Further studies including *in vivo* fertility and isolation and characterization of the alkaloidal principles with fertility potential are needed.

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Track 5, ID: ZIZIPHUS MAURITIANA- ROOT

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	0.06 Rf	0.3 AU	0.07 Rf	554.5 AU	36.10 %	0.12 Rf	36.3 AU	7778.6 AU	17.35 %	unknown *
2	0.15 Rf	68.5 AU	0.17 Rf	77.6 AU	5.05 %	0.19 Rf	39.8 AU	2113.7 AU	4.72 %	unknown *
3	0.25 Rf	80.1 AU	0.33 Rf	322.5 AU	21.00 %	0.38 Rf	34.7 AU	16634.2 AU	37.11 %	unknown *
4	0.38 Rf	95.1 AU	0.39 Rf	107.5 AU	7.00 %	0.42 Rf	35.5 AU	2332.4 AU	5.20 %	unknown *
5	0.48 Rf	35.1 AU	0.53 Rf	69.4 AU	4.52 %	0.55 Rf	53.0 AU	2872.9 AU	6.41 %	unknown *
6	0.58 Rf	64.3 AU	0.62 Rf	103.3 AU	6.73 %	0.63 Rf	39.4 AU	3481.2 AU	7.77 %	unknown *
7	0.65 Rf	89.7 AU	0.67 Rf	96.3 AU	6.27 %	0.69 Rf	33.1 AU	3364.0 AU	7.50 %	unknown *
8	0.70 Rf	83.3 AU	0.72 Rf	94.4 AU	6.15 %	0.75 Rf	75.0 AU	3266.8 AU	7.29 %	unknown *
9	0.75 Rf	74.6 AU	0.77 Rf	92.7 AU	6.03 %	0.80 Rf	2.0 AU	2872.7 AU	6.41 %	unknown *
10	0.83 Rf	0.9 AU	0.84 Rf	17.8 AU	1.16 %	0.85 Rf	0.8 AU	107.4 AU	0.24 %	unknown *

Figure 1: HPTLC Finger print of Ziziphus mauritiana scan wavelength 254nm

Group I	(Sterile water) served as control
Group II	Daily dose of extract (50mg/Kg) b.w. orally
Group III	Daily dose of extract (100mg/Kg) b.w. orally
Group IV	Daily dose of alkaloidal fraction (50mg/Kg) b.w. orally
Group V	Daily dose of alkaloidal fraction (100mg/Kg) b.w. orally

Table 1 : Grouping and treatment of animals

Parameters	Control group	ZM Extract treated group		ZM Alkaloidal fraction treated group	
		50 mg/Kg	100mg/Kg	50mg/Kg	100mg/Kg
Attraction toward females	14.16±1.07	17.5±1.2	18.5±1.2	20.6±1.5	23±1.6
PEI	23.0±2.2**	63.0±2.2**	64.1±2.3**	93±2.3**	95±2.4**
Mount intromission	0.33±0.21**	1.33±0.21**	1.44±0.22**	2.35±0.22**	2.38±0.23**
PEL	194.0 ±4.5**	146.3±4.4**	135±0.34**	140±3.2**	130±3.3**
IL	793.33±2.5**	191.63±1.0**	182±1.0**	99±1.5**	97±1.6**

Table 2: Sexual behavior studies

For each parameter, a minimum of five replicates were used and the results were expressed as Mean ± Standard Error (S.E.). All the data were analyzed under one way analysis of variance with least significant differences at $p < 0.01$.

Groups	Dose(mg/kg body weight)	Serum testosterone level (ng/mL)	Sperm count (million/mL)
Group I	Sterile water	0.93 ± .015	7.87 ± 0.24
Group II	50	1.14±0.17**	9.34 ± 0.26**
Group III	100	1.15±0.11**	9.77±0.25**
Group IV	50	1.56±0.90**	11.00±0.34**
Group V	100	1.75±0.88**	11.55±0.55**

Table 3: Serum Testosterone (mg/ml) level and sperm count in experimental male rats treated with extract(GroupII and III) and alkaloidal fraction(Group IV and V) of *Z. mauritiana* roots.

For each parameter, a minimum of five replicates were used and the results were expressed as Mean ± Standard Error (S.E.). All the data were analyzed under one way analysis of variance with least significant differences at $p < 0.01$